Effects of amended Soil & Light Conditions on Physiology & Metabolite Accumulation in *Chamaecostus cuspidatus* and *Mentha spicata* subsp. s*picata*

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Introduction

Human beings are dependent on plants for their several requirements such as food stuff, clothing, timber, medicine and various plant derived products. Medicinal plants have played a significant role since ancient times in human health and have been used as a source for various therapeutic agents to treat a variety of ailments. More than 3.3 billion people in developing countries utilize medicinal plants on a regular basis and are regarded as "backbone of traditional medicine" (Davidson et al., 2000; Robinson et al., 2011; Singh, 2015). Large number of important drugs used in the modern medicinal practices have been isolated or derived from plants (Dar et al., 2017). Medicinal properties of plants are due to the secondary metabolites present in them. These secondary metabolites do not play any fundamental or direct role in plant growth and metabolism, however they provide abiotic stress tolerance and are vital constituents of medicines (Akula et al., 2011).

Now a days, there is an increased interest in the public for consumption of plant based medicines as they are inexpensive, widely available and with minimum or negligible side effects (Skalli et al., 2019). Functional molecules from some medicinal plants have been discovered and identified as potential source of medicines while some already existing medicines are formulated from plant derived molecules (Rajendran et al., 2018). About 50,000 to 70,000 plant species are used in traditional and modern medicine (Perez et al., 2011).

India has a great variation in geographic and climatic conditions, soil texture and chemistry, water quality etc. and all these factors affect not only plant growth and yield but also their metabolism and production of phytoconstituents. Environmental stress is one of the biggest problems of this era. It is proved from various studies that production level and composition of phenolics and flavonoids varies according to the genotype of plant, climatic factors as, light intensity, relative humidity and temperature, environmental stimuli and agronomic practices (Ibrahim et al., 2012).

Abiotic stresses affect overall growth and other physiological and phytochemical processes in plants. Among them some of the abiotic stress can be controlled under greenhouse conditions thereby improving physiological and phytochemical processes of medicinal plants. Earlier studies have shown that light intensity can improve plant growth and alter their metabolite concentration for example, growth and photosynthetic capability of *Momordica grosvenori* was improved through increasing the

light intensity at a PPF (Photosynthetic Photon Flux) of 100 µmol $m^{-2}s^{-1}$ (Zhang et al., 2009). Although there are several environmental factors that affect photosynthesis and secondary metabolite production in plants but in field conditions various factors interact with each other for example light irradiance level generally interacts with high temperature and water deficiency. Thus, there is a need to further study and understand effect of individual and multiple environmental factors on physiology and phytoconstituents of medicinal plants (Yang et al., 2018).

Chamaecostus cuspidatus (syn. *Costus igneus* N.E.Br. and *C. cuspidatus* (Nees & Mart.) Mass (Specht et al., 2006; http://www.theplantlist.org/tpl1.1/record/kew-343822; https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?name=Costus%20igneus), commonly known as insulin plant, belongs to the family Costaceae. The plant is native to Southeast Asia and especially on the greater Sunda Island in Indonesia and has been recently introduced in to India (Krishnan et al., 2014). It is commonly grown as an ornamental plant in south India and is mainly cultivated in coastal areas of Karnataka and Tamil Nadu. Insulin plant is a full sun or partial shade loving plant and requires fertile soil and heavy moisture for proper growth and mostly planted near water (Hegde et al., 2014). *C. cuspidatus* is a perennial, upright, spreading plant. Leaves are simple, alternate, lanceolate, entire and petiolate. Flowers are orange and fruit is inconspicuous (Jyothi et al., 2015).

Several studies have shown that this plant possesses anti-oxidant, anti-diabetic, anti-inflammatory, antibacterial, antifungal, hypolipidemic, hypoglycemic and anti-cancerous activities (Mathew et al., 2019). These properties are due to the presence of several secondary metabolites as, flavonoids, terpenoids, carotenoids, tannins, saponins, alkaloids, glycosides, lignins, polysaccharides and phytosterols. Besides these compounds, corosolic acid an important triterpenoid is also found in insulin plant. Corosolic acid appears to have strong antidiabetic property and has been shown to improve insulin secretion and enhance glycolysis (Laha et al., 2019).

Mentha (Family: Lamiaceae) genus is widely distributed and highly evolved plant genus which is well known for its medicinal and aromatic value. *Mentha* is an important crop, mainly cultivated for the mint oil- a monoterpene, and is used in pharmaceutical, food, confectionery and liquor industries (Khanuja et al., 2006). This genus is mainly cultivated in India, Brazil, China, Europe

and America due to its immense economic value. Studies reported that mint oil has therapeutic potential as, antibacterial, antifungal, anti-oxidant, anti-inflammatory, diuretic, anthelmentic, stomachic, carminative, anti-spasmodic and local anesthetic (Sarer et al., 2011).

Mentha spicata subsp. *spicata* commonly known as pudina, spearmint, is a rhizomatous perennial, aromatic herb with medicinal value. It grows upto 30-100 cm in height has glabrous, erect and purplish stem. Leaves usually dark green are ovate to ovate-lanceolate, papery, with serrated margin and mucronate tip. Flowersare born on spike and purple (https://www.flowersofindia.net/catalog/slides/Pudina.html;http://www.efloraofgandhinagar.in/herb/mentha-spicata-subsp-spicata). The *Mentha* plant is rich in carvone, caffeic acid, rosmaric acid, luteolin, menthone, isomenthone, piperiton, piperitone oxide, piperitenone oxide, linalool, trans-subinene hydrate and 1,8-cineole (Gokbulut and Sarer 2010; Sarer et al., 2011).

Objectives

In view of the medicinal importance of the two plants *Chamaecostus cuspidatus* and *Mentha spicata* subsp. *spicata* have been identified to carry out a detailed study of the effect of two factors: soil stress, with respect to soil salinity and drought, and light conditions on their physiology and metabolite accumulation. The study will essentially focus on the influence of two environmental conditions on rate of photosynthesis and accumulation of secondary metabolite in these two plants.

The major objectives of the work will be:

1. Study effect of soil stress on physiology and secondary metabolite accumulation in the two selected plants.
2. Study effect of varying light conditions on physiology and secondary metabolite accumulation in the two selected plants.
3. Standardizing soil and light conditions for optimal physiological performance and secondary metabolite accumulation in the two selected plants.

Significance of the work

Due to urbanization salinity and drought are increasing and thus affecting environmental conditions of Agra and nearby regions. These factors along with temperature and light affect directly

and indirectly plant growth and development. Due to lack of awareness most of the medicinal plants are getting endangered and those found in sufficient number are not used conservatively. Cultivation of medicinal plants is also not practiced for a large number of species that are still collected from the wild. Thus the present study aims to accomplish the above mentioned objectives for the following expect deliverables:

1. *Chamaecostus cuspidatus* and *Mentha spicata* subsp. *spicata* will be established in Herbal Garden.
2. Correlation between selected abiotic stresses, photosynthetic efficiency and metabolite accumulation in these plants will be established.
3. Through standardization of a cultivation practice in stressed conditions we can improve the quality and quantity of pharmacologically active constituents.
4. We can utilize stressed conditions if found suitable to favor development of pharmacologically active constituents.
5. This work will also help to improve phytoconstituents in other plants.

Review of literature

Costaceae consists of four genera: *Costus, Monocostus, Dimerocostus* and *Tapeinochilos*. *Costus* is a large, polyphyletic genus. Three new genera are segregated from *Costus* and these are *Paracostus, Cheilocostus and Chamaecostus* (Specht et al., 2006).

Chamaecostus cuspidatus (syn. *Costus igneus* N.E.Br. and *Costus cuspidatus* (Nees & Mart.) (Specht et al., 2006; http://www.theplantlist.org/tpl1.1/record/kew-343822; https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?name=Costus%20igneus) is widely used for its anti-diabetic properties. For the first time in 2016, antiproliferative effect of *C. cuspidatus* and *C. subsessilis* was reported. The antiproliferative effect of these plants possibly linked to the apoptosis induction (Siquira et al., 2016). Leaf powder of *C. cuspidatus* was significantly decreased the blood glucose level in dexomethsobe induced diabetic rabbit (Deogade et al., 2018). They also compare *C. cuspidatus* with *Curcuma longa* and found its blood glucose lowering capacity more than *C. cuspidatus*. Ponnanikajamideen et al., (2018) synthesized gold nanoparticles using *C. cuspidatus* leaf powder extract and showed that the treatment of mice with these gold nanoparticles restored their

blood glucose, glycogen and insulin level.

Costus igneus is medicinal plants with lots of therapeutical importance. It is well known for its anti-diabetic activity. *C. igneus* have potential anti diabetic activity and it is proved from the study of Vishnu et al., 2010 that Ethanolic extract of *C. igneus* showed anti diabetic effect in alloxan induced diabetic rats. They compare the action of *C. igneus* extract with a well known anti- diabetic drug glibenclamide. These extract not only decrease the blood glucose level but also increased the body weight. Effect of *Costus igneus* leaves on hyperglycemia is proved by the study of Shetty et al., in 2010, they performed experiment on dexamethasone- induced hyperglycemia in male wister rats and found that the leaves of the above mentioned plant is effective in reducing the fasting and postprandial blood glucose level and bring them towards normal. They also compare their results with Glibenclamide tablets. Not only leaf but also its stem extract used pharmaceutically. For the first time the inhibitory effect of aqueous and ethanolic extract of *C. igneus* stem and isolated compound lupeol and stigmasterol on calcium oxalate urinary stones in albino Wistar rats were reported (Manjula et al., 2012). This study confirmed its inhibitory effect on stone (calcium oxalate crystals). Rhizome of this plant also has potential anti-diabetic and hypolipidemic activity. Quercetin and Kaempferol were isolated from the rhizome of *C. igneus* plant has anti-diabetic and hypolipidemic effect on streptozotocin (STZ) induced diabetic rats, they also compare their results with a standard reference drug glibenclamide (Kalailingam et al., 2013).

An insulin like protein were purified from *C. igneus* and this insulin like protein can decrease the blood glucose level when orally administrated, it didn't have any side effect when tested on mice. The name is given as insulin like protein because it doesn't have any structural similarity with insulin but function similarly (Joshi et al., 2013). A triterpenoid and a steroid compound had been isolated from *C. igneus* stem namely Lupeol and Stigmasterol respectively. These compounds were quantitatively estimated using high-performance thin-layer chromatography (HPTLC) method (Manjula et al., 2013). Including the above mentioned properties *C. igneus* also have anti-tumor activity, it have inhibitory effect on various human cancers including breast cancer. It is evaluated from a study conducted in 2014 that methanolic extract of *C. igneus* leaves have anti- proliferative and apoptotic effect on in-vitro MCF 7 Breast cancer cell line, this study also indicated that leaf extract

decrease tumor size without any effect on normal cells (Dhanasekaran et al., 2014).

Anti-inflammatory activity of the leaves of this plant is elucidated through a study; they isolated β-amyrin from *C. igneus* chloroform extract which serves as an anti-inflammatory component (Krishnan et al., 2014). In the series of isolation and characterization of compounds from *C. igneus*, an another compound diosgenin had been isolated from rhizome of the same pant and proved to be effective on cardiovascular risk, insulin secretion and β cell regeneration in STZ induced diabetic rats (Kalailingam et al., 2014). Methonolic extracts of leaf, stem and root of *C. igneus* shows potential antibacterial and antifungal activities and strongly inhibit the growth of all the tested bacteria and fungi in the study of Damam et al., 2017. Quercetin isolated from the leaves of *C. igneus* can be a useful therapeutic compound for diabetes. According to study quercetin and kaempferol have the best binding energies which activate the insulin receptor tyrosine kinase when compared with the standard drung known as pioglitazone (Peasari et al., 2018).

Recently ZnO nanoparticles from *C. igneus* leaves (Ci-ZnO NPs) were synthesized and they showed anti-diabetic and antioxidant properties more efficiently than its leaf extract. They also showed antibacterial and anti-biofilm activity against targeted bacteria (Vinotha et al., 2019).

Mentha spicata subsp. *spicata* is a potential herb having lots of medicinal and economic importance. RP-HPLC (Reverse Phase High Performance Liquid Chromatography) analysis of *Mentha spicata* subsp. *spicata* was done to determine some of the phenolic compounds in the extract of leaves of this plant. Phenolic compounds are mainly known for their antioxidant potential. This study showed that, along with caffeic acid and luteolin this plant is a good source of rosmarinic acid (Gokbulut and Sarer., 2010). A group of researchers identified thirty seven compounds from essential oil of *M. spicata* L. subsp. *spicata* using GC (Gas chromatography) and GCMS (Gas chromatography-mass spectrometry) analysis. The main constituents are carvone, 1,8-cineole, β-caryophyllene and trans-dihydrocarvone. This oil also possesses antimicrobial activity (Sarer et al., 2011). It is well documented that *Mentha sp*. has antioxidant potential. Among various species of *Mentha,* Benedec et al., (2013) made ethanolic extract of *M. viridis var. crispa*. And *M. longifolia* and evaluate their phenolic and flavanoid profile and found that in *M. viridis var. crispa*. Phenolic content is more and in *M. longifolia* flavonoids content is predominant. They also evaluated antioxidant potential in their

study. Moldovan et al., (2014) evaluated the antioxidant and antimicrobial activity of five different species of *Mentha* and found that they contained caftaric acid, gentisic acid, caffeic acid, p-coumaric acid, chlorogenic acid, rutin, isoquercitrin, luteolin and apigenin. A complete mass spectrometric analysis was done for phenolics and volatile compounds of *M. spicata* L. (spearmint) extract using ultra-high performance liquid chromatography- electrospray ionization-mass spectrometry (UHPLC-ESI-MS^n). In this study 66 compounds were identified which was the widest of that time (Cirlini et al., 2016).

Effect of different nutrient such as; N, P and K were well documented on different medicinal plants Potassium fertilization not only affect plant morphology but also their biochemical characters in *M. spicata*. Maximum amount of carvone content was observed in moderate level of potassium (325 mg/L) while large quantities of K increased polyphenols content and antioxidant activity (Chrysargyris et al., 2017).

In-vitro micropropagation followed by callus induction of *M. spicata* subsp. *spicata* was done using cotyledon node as explants. They tried 16 different combinations of NAA (α naphthaleneacetic acid) and BAP (6-Benzylaminopurine). After shooting and rooting they acclimatized rooted plants in green house (Ozdemir 2017).

Karaca et al., (2018) were isolated essential oil of *Lavandula stoechas* subsp. *stoechas* and *Mentha spicata* subsp. *spicata* and their components were determined using gas chromatography-flame ionization detector (GC-FID), gas chromatography/ mass spectrometry (GC/MS), thin layer chromatography (TLC). Major components of these essential oils were camphor and carvone respectively and their antibacterial activity against some common sinusitis pathogen was also tested. Effect of salinity on *M. spicata* L. was illustrated that highest salinity level(100 mM NaC) severely affect plant growth, photosynthetic rate, leaf stomatal conductance and total phenolics content of the plant, while low to moderate salinity level did not affect plant growth and biochemical processes. After the result of this study *M. spicata* could be predicted as a salt tolerant plant species which can tolerate moderate level of salinity (Chrysargyris et al., 2018).

Stress is a condition which is unfavorable to plant which directly or indirectly affect plant growth and metabolism. In case of medicinal plants we can use stress in favor to secondary metabolite

production because secondary metabolites produced in plants in defense against pathogenic attack and environmental stresses. This concept or idea is proved by several studies on various plants. A study conducted on tomato (*Lycopersicon esculentum*) to show the effect of exogenously applied brassionosteroids. They concluded that tomato plants grown in high temperature had decreased carboxylation efficiency, increased antioxidant activity and lipid peroxidation which downregulated activity of PSII. Pretreatment of EBR (24-epibrassinolide) can attenuate the inhibitory effect and mainly increased the activity of carboxylation and ROS scavenging system (Ogweno et al., 2008). The effect of light intensity on flavonoid concentration was shown by Ma et al., (2010) on *Anoectochilus formosanus.* They increased the light intensity from 10 to 60 µ mol m^{-2} s^{-1} which increases the superoxide dismutase activity resulting in increased accumulation of flavonoid concentration.

CO_2 enrichment increased the secondary metabolite production mainly total phenolics and flavonoids in *Labisia pumila*. However with the CO_2 enrichment and increased nitrogen fertilization production of the phenolics and flavanoids were decreased. This reduction could be due to accumulation of total nonstructural carbohydrate (TNC) and decrease in photosynthetic rate. This accumulation and reduction was indicated by increased the value of C:N ratio, photosynthetic nitrogen use efficiency and reduction in chlorophyll content (Ibrahim et al., 2011).

Irradiance level also affects secondary metabolite production as in case of *Orthosiphon stimaneus* Benth. In this plant secondary metabolite production enhanced under low irradiance level. This increased secondary metabolite production was related with increased production of CBSM (carbon based secondary metabolite) followed by TNC, H_2O_2 and MDA content (Ibrahim et al., 2012). Along with environmental factors fertilization and elicitors also affected secondary metabolism. Ibrahim et al., 2012 subjected *Labisia pumila* to different potassium fertilization and concluded that under highest potassium fertilization (270 kg k/h) net photosynthesis, stomatal conductance, intercellular CO_2, apparent quantum yield, soluble protein, total phenolics, flavonoids, ascorbic acid production, phenylalanine ammonia lyase activity increased while dark respiration decreased. A study reported that ABA enhanced the primary and secondary metabolite production in *Orthosiphon stimaneus* (Ibrahim et al., 2013). Like K fertilization, increased N levels in field of corn cultivars showed higher LDW (leaf dry weight), Fv/Fm (the maximum quantum yield of PSII), photosynthetic

CO_2 assimilation (Ph), leaf nitrogen concentration (LNC), leaf sugar concentration (LSC) (Jin et al., 2015).

In *Camellia sinensis* L Li et al; (2017) showed improved photosynthesis, respiration which eventually modified secondary metabolite biosynthesis under CO_2 enrichment. Nitric oxide (NO) also plays a significant role in plant development and reactive oxygen species homeostasis. Pan et al; (2019) used two mutants to show the effect of NO on leaf development and ROS activities.

Salinity is a major aspect in abiotic stress. Although salinity is a major threat to agriculture but depending upon its concentration low to moderate salt concentration in soil is good for plant growth and development. Asghari and Ahmadvand examined the effect of salt stress on *Aloe vera* and found its negative effect on plant morphology and biochemical characters which ultimately caused yield loss. They also demonstrated it as salt sensitive plant (Asghari and Ahmadvand 2018). Study showed that, salt stress not only changed plant growth but also antioxidant capacity, glandular trichome density and volatile exudates in *Schizonepeta tenuifolia*. The result of this study clearly showed that moderate salt stress increased antioxidants, phenolics and flavonoids (Zhou et al., 2018). Similarly moderate salinity (i.e., 120 mM of NaCl) increased the 20-hydroxyecdysone with no effect on photosynthesis and biomass accumulation in *Pfaffia glomerata.* This could be due to the upregulation of Spook and Phantom genes in plant (Felipe et al., 2019).

Proposed Methodology

Cultivation of plants

- Selected plants (*Chemaecostus cuspidatus and Mentha spicata* subsp. *spicata)* will be procured from CIMAP, Lucknow and other authenticated sources and established in the herbal garden of DEI.
- Plants will be grown in both controlled and stressed conditions.
- Pot experiments will be designed and plants will be grown in greenhouse under controlled and stressed conditions.
- Plants will be subjected to different levels of the following conditions:
    1. Light intensity
    2. Salinity

3. Drought

- Soil analysis will be done and nutrient dose will be given according to plant requirement.

Physiological analysis

- **Growth parameters**- plant height, plant fresh and dry weight, plant biomass will be measured.
- Photosynthetic rate, leaf gaseous exchange and fluorescence will be measured.
- Determination of total chlorophyll will be carried out using method described by Yang et al. (2016).

Biochemical analysis

- Enzymatic assay will be done for Superoxide dismutase (SOD), Catalase (CAT) and Peroxidase (POD) as per the method used by Felip et al. (2019).
- Proline content will be determined as per the method used by Felip et al. (2019).

1. **Biochemical analysis of *Chemaecostus cuspidatus***

- Plant extract will be prepared using appropriate solvent (for example-methanol or ethanol).
- Qualitative analysis of leaf extracts of *C. cuspidatus* for flavonoids, alkaloids, tannins, terpenoids, saponins will be done by using the method of Peasari et al. (2018).
- Quantification of a particular secondary metabolite will be done using chromatographic methods (HPLC or HPTLC methods).

2. **Biochemical analysis of *Mentha spicata* subsp. *spicata***

- Plant extract or essential oil will be prepared as per the experimental requirement.
- Quantitative estimation of essential oil and other compound of interest will be done using chromatographic methods (HPLC or GC-MS methods).

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